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Behavior of Legume Bacteria (*Rhizobium*) in Relation to Exchangeable Calcium and Hydrogen Ion Concentration of the Colloidal Fraction of the Soil

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THOMAS M. MCCALLA*

INTRODUCTION

The study of soil microorganisms has long been handicapped by the difficulty of separating their complex soil environment into known and controllable conditions. It has been recognized[24 25]† that as a bacterial medium the soil is different from any improved nutrient solution. In the soil, the ions are adsorbed on the soil complex with a very low concentration in the soil solution; on the contrary in a nutrient solution they are free to diffuse through the medium and may be present in concentrations sufficient to be injurious. Thus, any deductions regarding the behavior of macro- or microorganisms under conditions of a nutrient solution can be applicable to those of the soil only with reservations.

Legume bacteria‡ must live from four to six months annually and even longer in the soil away from their host. They obtain their energy under such conditions from previously elaborated organic materials, and their inorganic nutrients largely from the soil's colloidal fraction, which is of primary importance in making them available. Very little information exists regarding the status, behavior and reproduction of the legume bacteria during this period of absence of this host plant, when they must subsist on nutrients delivered by the soil complex.

Resolution of the complex soil environment into known and controllable conditions necessarily involves the use of some major constituent of the soil which is of primary importance in controlling the mineral balance. Since the soil colloidal fraction is designated as the constituent largely responsible for the physical and

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†Numerals indicate "References," page 43.

‡The terms "legume bacteria" and "Rhizobium" will be considered synonymous and used interchangeably.

chemical behavior of a soil, and since it is also of primary importance in controlling and maintaining both the supply and the balance of nutrients for higher plants, it was selected as a carrier of mineral nutrients for the legume bacteria in this study. Such a soil colloidal system which contains a known balance of mineral nutrients adsorbed on its complex should simulate the natural soil habitat of the microorganism sufficiently so that results obtained with such a system would represent those of the natural soil conditions in the field very closely.

Recognition of the legume bacterium as a colloidal matrix of protoplasm encased in a delicate yet rigid cell membrane with an outer surface that is significantly small though immensely larger than the soil colloid, gives a substantial foundation for a better understanding of the innumerable life processes that occur in the intimate association of the water film of the colloid and the bacterial cell. A fundamental understanding of the nutrition of the legume bacteria can be reached only by observations made on these organisms in a controlled natural habitat. It was in this belief that an attempt was made in this study to use the colloidal fraction of the soil as an environment permitting careful chemical control of the mineral nutrients, in order to learn thereby of the relation of exchangeable calcium and hydrogen to the behavior of legume bacteria, or Rhizobium.

HISTORICAL

Variation in colony form and color, effective and ineffective strains, failure to nodulate the host, and abnormalities of other descriptions have been observed frequently in legume inoculation practices, both in the laboratory and field. These conditions have been associated with various chemical or physical effects of the environment or with chemical by-products during the growth of the organism. As a consequence of these uncommon manifestations, complex life cycles have been ascribed to the legume bacteria as a provocation of environment or as inherent in the nature of the organism.

That different cations are stimulative in the growth of microorganisms and that such cations also exert certain effects on each others have been reported by various workers.[20, 30] Winslow et al [36, 37] in their extensive research on the effects of cations on the viability of Bac. coli found that all cations stimulated growth in certain concentrations. Hotchkiss'[27] experiments indicated that Bac. coli was more responsive to calcium and magnesium than to other cations. Many workers have studied the optimum hydro-

gen ion concentration for microorganisms[19, 31] Fellers,[21] working with soybeans, noted that the bacterial infection of the roots did not take place readily in acid soils, even when a good supply of bacteria was present.

That mutation of microorganisms occurs under certain circumstances has been attested to by recent investigators.[12, 18, 34] Rettger and Gillespie[32] demonstrated the influence of oxygen starvation in developing variant forms of Bacillus megatherium. When potassium permanganate was added, growth was restimulated. B. Coli[14] gave variant forms and was maintained in this condition by frequent transfers in broth. Variant forms were considered as an adaptation of the organism to the environment.

The importance of calcium has been widely appreciated. Almon and Baldwin[11], in a study of variant legume bacteria, found that variant forms could be converted to normal (agencies not specified) which would nodulate the host. When normal legume organisms were put into an acid soil they became brown, gummy and yellow. Foote, Peterson, and Fred[22] presented evidence that the addition of calcium carbonate to culture media increased fermentation. Horner and Burk[26] stated that in the absence of calcium the growth of azotobacter decreased fifty per cent. They concluded that calcium acts in a truly catalytic role in nitrogen fixation.

Albrecht et al[1, 6, 7, 8] have shown that lime is essential for successful legume crops, not merely as a neutralizing agent but also because its calcium serves as a nutrient. Calcium may be supplied to the plant through salts with no acid neutralizing effect or through small applications of fine lime without completely neutralizing the soil.

Albrecht and Davis[2] have shown that there is a distinct difference in the cell wall structure of a calcium fed plant and one that did not have calcium. The latter had a structure that was easily broken, while the calcium-bearing soybean section retained its structure.

Albrecht and Jenny[3] found that a growth irregularity resembling the "damping off" disease was closely associated with a shortage in the supply of available calcium. When the calcium supply was high the apparently diseased condition did not occur.

Albrecht suggested[5] that the requirement of the plant for calcium must first be met and then an additional amount added to permit nodulation. He showed further[4] that the sensitivity of the plant to soil reaction is greater as the calcium supply is less. Calcium proved to be two and one-half times more significant in

growth than the degree of acidity. Calcium was about one and one-half times as influential on nodule numbers as the hydrogen-ion concentration.

Scanlan[33] suggested that legume cultures treated with calcium were more viable than those stored without calcium. Cameron and Sherman[17] found that the legume bacteria would grow better in contact with a calcium carbonate agar layer in liquid media than in a liquid medium without this contact.

These observations on the varied effects of the different cations, media, and conditions on bacterial mutation, behavior, and variation suggest that certain nutritional deficiencies may be responsible for the appearance of abnormal forms in the cultures. The influence of calcium on legume plant growth and on nodule producing bacteria suggests such a close association between calcium and successful nodulation that its deficiency may be responsible for the appearance of abnormal or mutation forms. The test of this latter point is the main theme of this study.

EXPERIMENTAL

Plan and Method of Experiment

In order to understand and appreciate more fully the relationship of the legume bacteria and host to calcium, this study is divided into two phases: first, the effect of calcium on the legume bacteria as it modifies the variant and normal, and second, the function of calcium through the host by influencing nodulation when variant and normal bacterial forms are used as inoculants.

Reasons for Particular Media Used.—Because of accumulated knowledge about the chemical behavior of the colloidal fraction of Putnam silt loam, accurate prediction can be made as to its behavior with various cations. Because it is the chemically-active fraction of the soil, whose properties and behavior are fairly well known, it was selected to serve as a soil base on which essential nutrients for the legume bacteria would be adsorbed. Although adsorbed nutrients are insoluble in water, yet they are available to plants[28] and should likewise be usable by bacteria.

History and Growth Characters of Cultures Used.—A series of variant, or abnormal legume bacteria, was obtained through the courtesy of A. J. Hofer, Geneva, New York, state bacteriologist for the inspection of commercial legume inoculants, who had isolated them from reliable cultures of undoubted purity. These variants were used as test specimens for the relative effect of calcium-bearing and non-calcium media. For comparison with the

behavior of these variant or abnormal organisms, some normal legume bacteria that had been tested for their nodulation of the host

TABLE 1.—HISTORY OF CULTURES USED.

Culture Number	Legume Species	Color of Pigment Production	Colony Appearance	Original Source or Author
		Abnormal or Atypical Cultures		
V-2	Alfalfa	Yellow	Scant growth	Baldwin
V-10	Alfalfa	Yellow	Heavy growth	Hofer
V-11	Alfalfa	Pink	Scant growth	Hofer
		Normal or Typical Cultures		
N-1	Alf-2	White	Heavy growth	Missouri Lab.
N-5	Alf-2	White	Heavy growth	Missouri Lab.
N-6	Soy-502	White	Scant growth	Missouri Lab.
N-7	Alf-3	White	Heavy growth	Missouri Lab.

and which showed the general characters of the particular group to which they belonged were used. The general cultural characters of these organisms on the regular laboratory medium are listed in Table 1. Alfalfa and Virginia soybean were used as the test plants. They were grown under greenhouse conditions in sand media during the season of the year when conditions were favorable.

Preparation of Colloidal Media

Colloidal Clay Preparation.—A hydrogen clay was prepared by the method of Bradfield[15, 16] by churning the heavy subsoil of Putnam silt loam, siphoning off the suspension, centrifuging in a super-centrifuge, collecting the dark, greasy upper por-

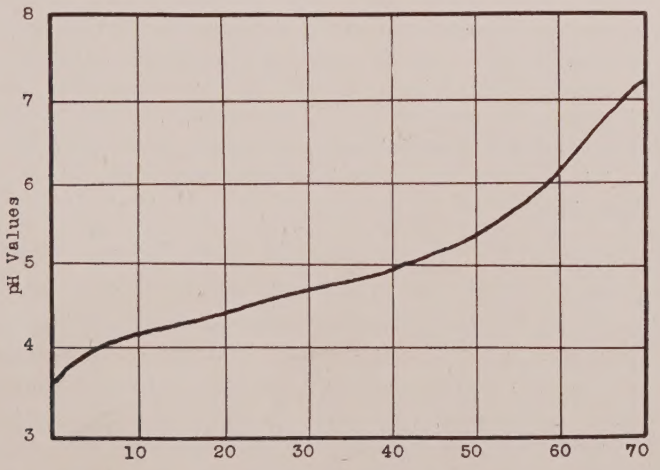


Fig. 1.—Calcium hydroxide titration curve. M.E. of calcium hydroxide per 100 grams of clay.

tion from the celluloid sleeve of the centrifuge and dialyzing under an electric current of 110 volts until the colloid was free of bases. The hydrogen clay behaved as shown in Figure 1 when titrated with calcium hydroxide.[13]

Nutrients Added to Clay.—In planning a medium which would contain the essential nutrients for legume bacteria, the regular laboratory medium served as a guide. The contents of the regular and proposed media are listed in Table 2. The only

TABLE 2.—MINERAL CONTENT OF THE CUSTOMARY AGAR AND THE COLLOIDAL CLAY MEDIA.

Reagents in Customary Medium	M.E. per Liter	Reagents in Colloidal Medium	M.E. per Liter	Lbs. per 2 million
Sucrose	10 gms.	Sucrose	10 gms	----
NaCl	3.34	K	2.66	8299
K_2HPO_4	8.62	PO_4	4.00	3200
$MgSO_4$	3.33	MgO	2.00	1920
$CaCO_3$ (soluble)	1.29	$Ca(OH)_2$	11.93	19088
Yeast Solution	100 ml.	Kraut Juice	20 ml.	----
Water	900 ml.	Water and colloid	980 ml.	----

significant difference between the two was the amount of available calcium. There was a potential supply of 60 M. E.* of calcium in the customary calcium carbonate medium, of which only 1.29 M. E. were soluble or available, while in the colloidal clay medium there were 11.93 M. E. of available calcium. Thus, there was ten times as much available calcium in the colloidal clay medium as in the calcium carbonate medium.

It would be possible to supply calcium in solution by using some soluble calcium salt in quantities equal to the concentration obtained in the calcium colloidal clay, but the ionic calcium at this concentration might be injurious. When the calcium is supplied through the colloidal clay, the latter acts as an inert anion for the calcium and a large supply of adsorbed but unionized calcium is provided.

In the preparation of the colloidal media, known amounts of the clay were titrated with known amounts of phosphorus in the phosphate or oxidized form as an anion, and of magnesium, potassium, calcium and other cations until brought to the saturation point as indicated by reaction changes. The titration curves of these various mineral nutrient additions are represented graphically in Figure 2.

*M.E. represents milligram equivalents.

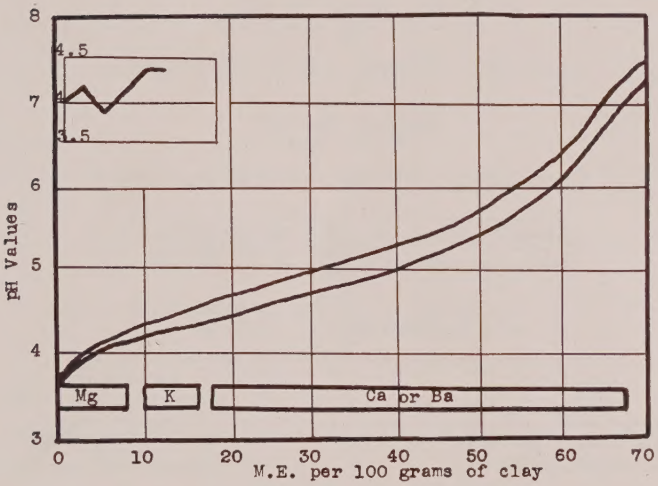


Fig. 2.—Reaction curves of the preparation of colloidal clay by the addition of mineral nutrients.
Upper curve—Ca-K-Mg-Clay titration
Lower curve—Calcium titration
Insert—pH variation when 16 M.E. PO_4 were added

Preparation of Bacteriological Media.—The bacteriological medium determined upon was one with a total adsorption capacity of 16.5 M. E. of cations per liter by 25 grams of clay per liter (66 M. E. per 100 grams clay). This was then a 2.5% clay suspension. The preparation of the colloidal medium as outlined in Figure 3 consists of the following procedure: Dry magnesium oxide (0.0403 gms.) was added to the hydrogen clay at the rate of 2 M. E. per liter (or 8.00 M. E. per 100 grams of clay), stirred and allowed to react for two days. Dipotassium-phosphate was next added in the amount of 4 M. E. (or 16 M. E. per 100 grams of clay) of the phosphate ion and 2.66 M. E. (or 11 M. E. per 100 grams of clay) of potassium. After sufficient time had elapsed for reaction to take place, the solution had a pH of 4.15. When the magnesium, potassium and phosphate clay was washed through a filter paper coated with colloidon in the ultra filter under a pressure of 100 pounds, 1.47 M. E. of the 16.0 M. E. of the phosphate ion added were removed in the clear aqueous filtrate. The pH value of the resuspended clay thus rose to 4.55. This clay had 12.85 M. E. of available[35] and 13.75 M. E. of electrodialyziable phosphate per liter of the material when brought back to the original volume by the addition of distilled water.

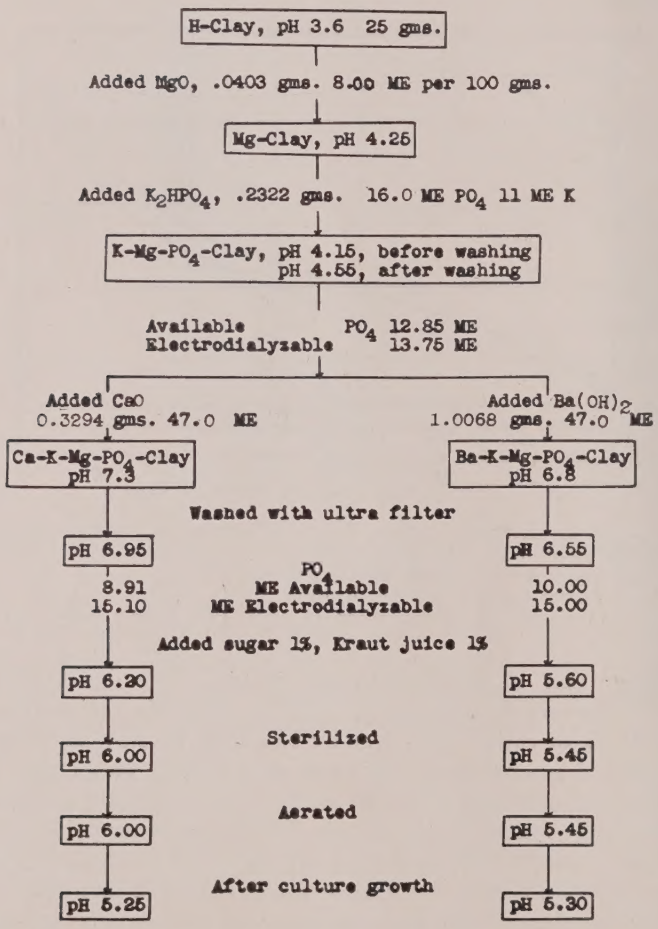


Fig. 3.—Scheme of preparation of media with their behaviors

The magnesium, potassium and phosphate colloidal clay was divided into two equal parts. Calcium was added as calcium hydroxide to one portion and barium as the hydroxide to the other. Addition of the calcium hydroxide brought the reaction to a pH value of 7.3. After washing in the ultrafilter the amount of electrodialyzable phosphate was 15.1 M. E. and the available phosphate, according to the Truog test, 8.91 M. E. per 100 grams of the clay. Barium hydroxide, added to the other fraction, brought the pH value to 6.8. Subsequent washing through

the ultrafilter gave a barium, magnesium, potassium and phosphate clay with an available phosphate content of 10.0 M. E. and an electrodialyzable amount of 15.0 M. E. per 100 grams of clay.

One per cent sucrose solution and 1% kraut juice were then added to each of these colloidal clays. After they were sterilized for 20 minutes at 15 pounds pressure, the pH value fell to 6.00 for the calcium clay; for the barium clay under the same conditions the figure was 5.45.

Behavior of Clay System on Addition of Phosphorus.—When dipotassium phosphate was added to a magnesium clay initially at a pH of 4.15 and the pH value measured daily for about ten days, the figures obtained were irregular. They fluctuated above and below the initial value erratically. No reasons for the variations are ventured.

The Separate Effects on Colloidal Media by Kraut Juice, Sugar, Sterilization and Aeration.—In order to ascertain the factors responsible for the changes in the degree of acidity of the media during their preparation, the addition of each unit was made to the media separately and the pH change observed. Two media were prepared according to the plan outlined previously, one as a *calcium*-magnesium-potassium-phosphate clay and one as a *barium*-magnesium-potassium-phosphate clay. Each of these was brought to the cation saturation capacity. Without the addition of any other item to these media, they were autoclaved and aerated. Sterilization lowered slightly the pH of both the calcium and barium complexes. Possibly the higher temperature caused the reaction between the colloid and bases to go more nearly to completion. Upon aeration of these complexes no change was observed in the pH value.

The addition of 0.25 grams of sucrose, ½ ml. of kraut juice per 25 ml, of colloidal clay for each medium lowered the initial pH value considerably. The sterilization and aeration of these media resulted in no material change in the pH value of either the calcium or the barium clay complex. These itemized changes are listed in Table 3.

The addition of ½ ml. of kraut juice seemed to lower the pH value more than was true for sterilization or the addition of sugar. Aeration had no effect on the pH. This would suggest that carbon dioxide which is contained in the air does not react with the calcium of the clay to form calcium carbonate, which reaction would bring about a change in pH. Thus with no reaction change in the media,

TABLE 3.—INFLUENCE OF KRAUT JUICE, SUCROSE, STERILIZATION AND AERATION ON THE pH OF COLLOIDAL CLAY.

	Media	Sterilization		Aeration
		Before	After	
1	$Ca\text{-}Mg\text{-}K\text{-}PO_4$-Clay	7.40 7.40	7.20 7.20	7.20 7.20
2	$Ba\text{-}Mg\text{-}K\text{-}PO_4$-Clay	6.90 6.90	6.85 6.85	6.85 6.85
3	$Ca\text{-}Mg\text{-}K\text{-}PO_4$-Clay and 0.5 ml. Kraut Juice	5.85 5.85	6.10 6.10	6.10 6.10
4	$Ba\text{-}Mg\text{-}K\text{-}PO_4$-Clay and 0.5 ml. Kraut Juice	5.10 5.10	5.25 5.25	5.40 5.40
5	$Ca\text{-}Mg\text{-}K\text{-}PO_4$-Clay and 0.25 grams Sucrose	7.00 7.00	7.10 7.10	7.00 7.00
6	$Ba\text{-}Mg\text{-}K\text{-}PO_4$-Clay and 0.25 grams Sucrose	6.55 6.55	6.30 6.30	6.20 6.30
7	$Ca\text{-}Mg\text{-}K\text{-}PO_4$-Clay, 0.25 grams Sucrose 0.5 ml. Kraut Juice	6.20 6.20	6.30 6.30	6.30 6.30
8	$Ba\text{-}Mg\text{-}K\text{-}PO_4$-Clay, 0.25 grams Sucrose 0.5 ml. Kraut Juice	5.50 5.50	5.65 5.65	5.65 5.65

the calcium must remain adsorbed and the bacteria must secure it from the clay rather than through any solubility phenomena.

Colloidal Clay Media with Variable Calcium Levels.—In order to prepare clays with various levels of calcium, it was necessary to substitute other cations to complete the saturation requirement of the clay. Such cations as hydrogen, barium, potassium, and magnesium were used. These clays were prepared to observe the effect of the degree of saturation of the clay with calcium on the legume organism.

Analytical Methods

Methods of Bacteriological Analysis.—The sterile, colloidal clay suspensions of completed media were put into 100 ml. test tubes in 25 ml. quantities, inoculated with the legume organisms to be tested and then aerated with air that had been washed with water and dried in sulfuric acid. The air was forced through the clay suspension at a sufficient rate to keep bubbles passing continually. The cultures were maintained at room temperature of 25° to 30° C. After aeration for three days, they were plated out on yeast-mannitol agar carrying 5 ml. of 0.5 per cent brom-thymol blue per liter. Loop dilutions were used in making the plates.

When the colonies appeared on the plate, their character and numbers were studied and tabulated. Percentages of typical and

atypical forms were observed as an index of the effect of the calcium and the no-calcium bearing media in changing the characters of the variant and normal legume organisms. Consideration was given to the acid reaction induced by the organisms in changing the blue agar to a yellow which made the organisms appear yellow upon a superficial examination. Variant and normal forms were selected and transferred to customary calcium carbonate slants for repetition of the process. Details for this procedure are more fully outlined in Figure 4. The organisms obtained by growing the variant and normal forms each in the calcium and no-calcium media were tested for their ability to nodulate the host growing in the presence or absence of calcium.

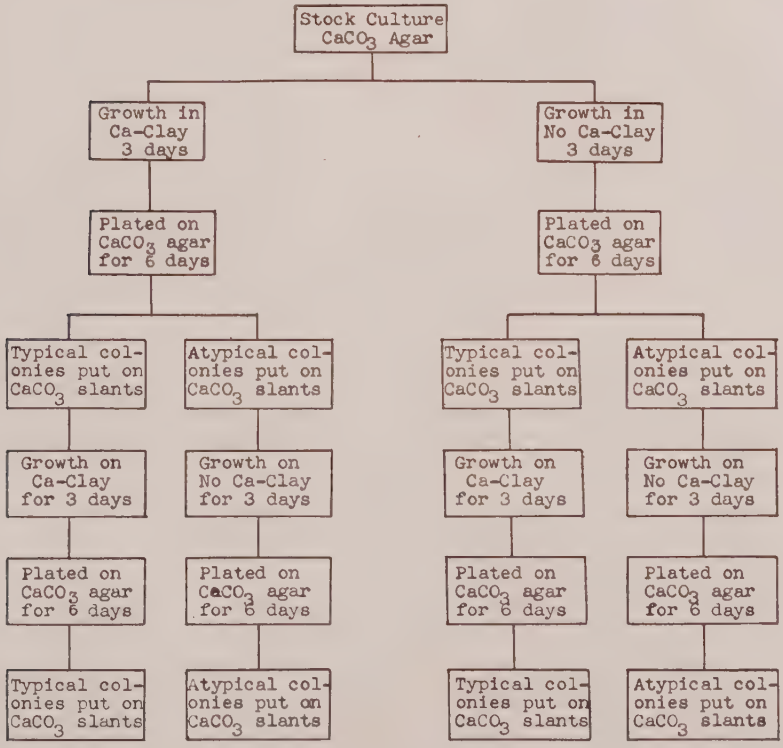

Stock Culture $CaCO_3$ Agar
Growth in Ca-Clay 3 days
Growth in No Ca-Clay 3 days
Plated on $CaCO_3$ agar for 6 days
Plated on $CaCO_3$ agar for 6 days
Typical colonies put on $CaCO_3$ slants
Atypical colonies put on $CaCO_3$ slants
Typical colonies put on $CaCO_3$ slants
Atypical colonies put on $CaCO_3$ slants
Growth on Ca-Clay for 3 days
Growth on No Ca-Clay for 3 days
Growth on Ca-Clay for 3 days
Growth on No Ca-Clay for 3 days
Plated on $CaCO_3$ agar for 6 days
Plated on $CaCO_3$ agar for 6 days
Plated on $CaCO_3$ agar for 6 days
Plated on $CaCO_3$ agar for 6 days
Typical colonies put on $CaCO_3$ slants
Atypical colonies put on $CaCO_3$ slants
Typical colonies put on $CaCO_3$ slants
Atypical colonies put on $CaCO_3$ slants

Fig. 4.—Scheme of growing cultures.

Methods of Chemical Analysis.—Standard methods were used for all chemical work. The pH was determined by the quinhydrone electrode. Phosphorus was determined by the colorimetric method.[35]

Results

Growth of Legume Bacteria in Colloidal Clay Solutions Saturated with Calcium Paired with Other Cations.

Calcium and Barium.—Five clay suspensions were saturated with calcium and barium, with calcium in increasing amounts and barium correspondingly less. To each of the 25 ml. suspensions there were added 0.25 grams of sucrose and 0.5 ml. of yeast extract (prepared by autoclaving 10 grams of dried yeast in one liter of distilled water). These were sterilized for twenty minutes at 15 pounds pressure. The suspensions were inoculated with a drop of a fresh culture of legume bacteria and aerated for five days. The number of bacteria was determined by the usual dilution and plating method. The degree of saturation of H-clay with calcium, the number of bacteria and the pH of the media before and after growth of the bacteria are listed in Table 4 and given graphically in Figure 5.

Normal legume bacteria were used for the tests. A typical soybean organism, N-6, grew best in the clay which contained a large amount of calcium, although there was fair growth in the clay wholly saturated with barium. This suggests that legume bacteria may use barium as a nutritive element and that it is not toxic. The same growth response was obtained with a typical alfalfa culture, N-5. The alfalfa culture may use barium as a nutritive element but it is not as well used nor does it give as great a growth as calcium. It may be possible that there are sufficient calcium and other minerals in the yeast extract to help the bacteria to grow in the barium clay.

TABLE 4.—NUMBERS OF LEGUME BACTERIA IN RELATION TO AMOUNTS OF CALCIUM AND BARIUM IN THE MEDIA.

Culture Used	No.	ME. per 100 gms. Clay		pH		Bacteria per ml. (millions)
				Before	After	
		Calcium	Barium	Colony Growth		
N-6	1	0.0	70.0	6.70	6.86	65.0
	2	20.0	50.0	6.90	7.35	97.0
	3	40.0	30.0	7.10	7.30	226.8
	4	50.0	20.0	7.20	7.40	145.8
	5	70.0	0.0	7.30	7.45	156.8
N-5	1	0.0	70.0	6.70	6.00	3.6
	2	20.0	50.0	6.90	6.45	4.8
	3	40.0	30.0	7.10	6.60	6.6
	4	50.0	20.0	7.20	6.50	32.4
	5	70.0	0.0	7.30	6.40	43.2

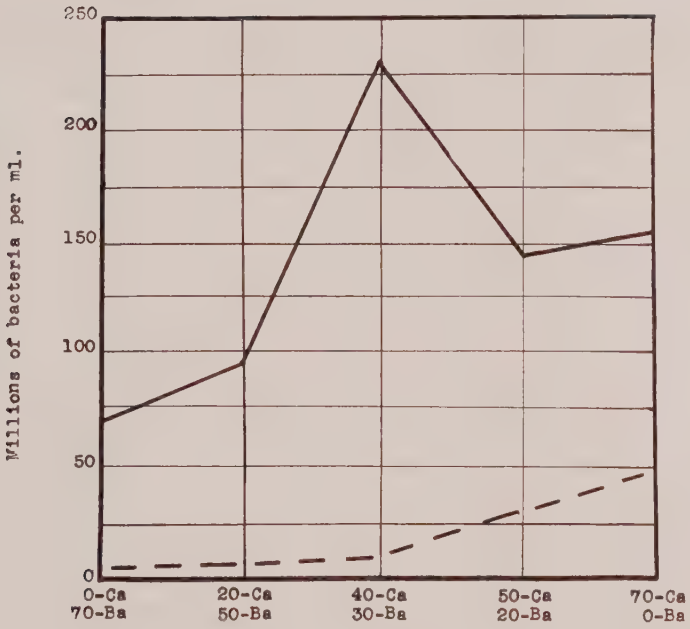


Fig. 5.—Bacterial numbers as related to the M.E. of calcium and barium per 100 grams soil.
Upper curve—for normal culture N-6
Lower curve—for normal culture N-5

Calcium and Hydrogen.—This clay was prepared as were the barium and calcium clays with the exception that two additional saturations were included. Six clays were prepared, varying from 0 to 70 M. E. in increments of 10 and 20 M. E. of calcium and hydrogen. One additional clay was prepared which contained 80 M. E. of calcium. This gave six clays containing different amounts of calcium and a reciprocal amount of hydrogen, and one clay which contained calcium in excess of saturation. To these clays were added 0.25 grams sucrose and 0.5 ml. of yeast extract per 25 ml. They were autoclaved, inoculated, aerated for five days and then plated for counts and observations.

It was observed that for 40 M. E. and less of calcium per 100 grams of clay there were only slight growths on the plates with dilutions less than one to a million. The colonies occurring were yellow. In the clays containing 50 M. E. of calcium per 100 grams of clay there was fair growth. As the amount of calcium increased, the number of bacteria increased and the colonies were white. The results are given in Table 5 and Figure 6.

TABLE 5.—NUMBERS OF LEGUME BACTERIA IN RELATION TO AMOUNTS OF CALCIUM AND HYDROGEN IN THE MEDIA.

Culture Used	No.	M.E. per 100 gms. Clay		pH		Bacteria per ml. (millions)
				Before	After	
		Calcium	Hydrogen	Colony Growth		
N-6	1	0.0	70.0	3.60	3.55	0.0
	2	20.0	50.0	4.35	4.15	0.0
	3	40.0	30.0	5.10	4.40	0.0
	4	50.0	20.0	5.35	5.40	30.0
	5	60.0	10.0	6.25	5.70	130.0
	6	70.0	0.0	7.25	7.20	150.0
	7	80.0	0.0	7.85	7.85	305.0

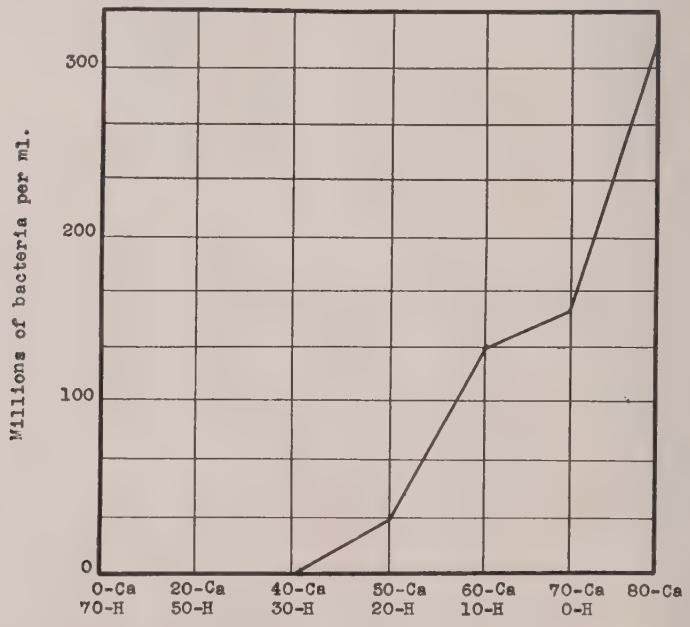


Fig. 6.—Bacterial Numbers as related to the M.E. Calcium and Hydrogen per 100 gms. of Clay.
Culture N-6

From these results it is apparent that in the absence of bases, or liberal presence of hydrogen, the organism does not receive the required amounts of nutrients and cannot make its growth. It would then appear that it is not correct to say that the detrimental effect of hydrogen ion caused the lack of growth; on the contrary, it seems evident that it is the absence of bases that has resulted in this failure. The growth of normal soybean organisms, N-6, produced a more acid reaction in the medium. If the medium was made more acid by the microorganisms, hydrogen was introduced. This was possible on this saturated clay only by calcium

removal. This calcium removal must have been brought about by the microorganisms and we can believe them capable of satisfying their cation nutrient requirements by taking such off the clay in the adsorbed form by exchanging hydrogen for them.

Calcium and Potassium.—The calcium and potassium clays were prepared in manners similar to those for the clays mentioned previously. There were five calcium and potassium clays saturated at different levels of 10 and 20 M. E. increments. One-fourth gram of sucrose and 0.5 ml. of yeast extract per 25 ml. were added to these clays. They were autoclaved, inoculated, aerated and plated.

A typical soybean culture, N-6, grew well even in the clay completely saturated with potassium. However, as the calcium increased and potassium decreased, the growth gradually increased. The clay completely saturated with calcium grew twice as many bacteria as the one completely saturated with potassium. This again emphasizes the fact that even though potassium may be utilized as a nutritive element and the organisms may make a growth

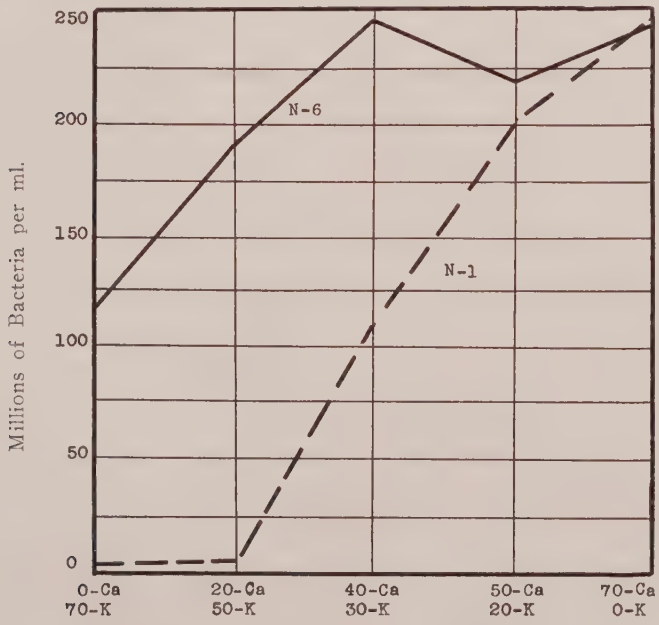


Fig. 7.—Bacterial numbers as related to the M.E. of Calcium and Potassium per 100 grams Clay.
Upper curve—N-6—a normal culture
Lower curve—N-1—a normal culture

on this element alone, growth is very much better when calcium is supplied in the proper amount.

A typical alfalfa organism, N-1, did not make satisfactory growth in the clays saturated 70% and 100% with potassium. When the calcium saturation exceeded 30% the organism made good growth. The results are given in Table 6 and Figure 7.

TABLE 6.—NUMBERS OF LEGUME BACTERIA IN RELATION TO AMOUNTS OF CALCIUM AND POTASSIUM IN THE MEDIA.

Culture Used	No.	M.E. per 100 gms. Clay		pH		Bacteria per ml. (millions)
				Before	After	
		Calcium	Potassium	Colony Growth		
N-6	1	0.0	70.0	7.25	6.85	120.0
	2	20.0	50.0	6.35	6.50	190.0
	3	40.0	30.0	7.10	6.60	250.0
	4	50.0	20.0	6.80	6.85	220.0
	5	70.0	0.0	6.80	6.70	240.0
N-1	1	0.0	70.0	7.25	6.70	0.21
	2	20.0	50.0	6.35	6.25	0.35
	3	40.0	30.0	7.10	6.45	120.0
	4	50.0	20.0	6.80	6.00	216.0
	5	70.0	0.0	6.80	6.25	250.0

Calcium and Magnesium.—The calcium and magnesium clays were prepared by saturating hydrogen clay with calcium hydroxide and magnesium oxide in different amounts as shown in Table 7. Sucrose in the usual concentration and 0.5 ml. of yeast per 25 ml. of clay suspension were added. These clays were autoclaved, inoculated, aerated and plated for colony counts and color observation.

TABLE 7.—NUMBERS OF LEGUME BACTERIA IN RELATION TO AMOUNTS OF CALCIUM AND MAGNESIUM IN THE MEDIA.

Culture Used	No.	M.E. per 100 gms. Clay		pH		Bacteria per ml. (millions)
				Before	After	
		Calcium	Magnesium	Colony Growth		
N-7	1	0.0	70.0	6.80	6.55	64.0
	2	20.0	50.0	6.00	4.70	64.8
	3	40.0	30.0	6.70	6.25	124.2
	4	50.0	20.0	6.80	6.80	135.0
	5	70.0	0.0	6.00	5.53	129.6
N-6	1	0.0	70.0	6.80	6.20	169.0
	2	20.0	50.0	6.00	4.85	300.0
	3	40.0	30.0	6.70	6.8	450.0
	4	50.0	20.0	6.80	7.15	450.0
	5	70.0	0.0	6.00	5.75	300.0

The normal alfalfa organism, N-7, grew well in all concentrations of magnesium and calcium. An increase of calcium, however, gave an increasingly better growth. Magnesium seemed to

function as a nutritive element with an ability to provoke growth response not widely separated from that of calcium. With the normal soybean organism, N-6, the same occurred with respect to growth response. The number of bacteria occurring on the various magnesium and calcium clays is given in Table 7 and Figure 8.

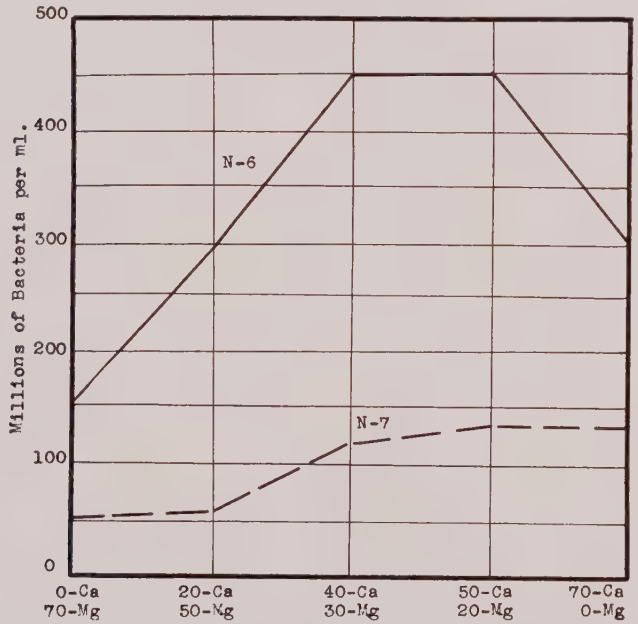


Fig. 8.—Numbers of legume bacteria as related to the M.E. of Calcium and Magnesium per 100 grams Clay.
Upper curve—N-6—a normal culture
Lower curve—N-7—a normal culture

In certain concentrations of magnesium and calcium there was better growth than in calcium alone. Thus, the presence of magnesium with calcium has increased the number of bacteria. It has been observed that these salts, when used together, increased the length of viability of legume bacteria.[10]

The pH value of the clays varied considerably. It was observed that the growth increased with increasing calcium and not with changing pH values. Thus, there seems to be no relation here between pH and growth, while there is a definite relation between growth and the amount of calcium.

Growth Behavior of Legume Bacteria in Clays Containing a More Complete Supply of Nutrients.

Nutrients More Complete but Deficient in Calcium.—In the previous cultures the growths were made in colloidal clay media supplying only two principal cations, of which calcium was one. In order to determine more carefully the effect of the presence or absence of calcium on the variant forms and on the normal forms of Rhizobium, more of the essential nutrients were added to the media. Thus two media, one with *calcium,* potassium, magnesium and phosphate on the clay, and the other with *barium,* potassium, magnesium and phosphate on the clay were prepared as previously outlined in Figure 3. The barium, which is closely related to the calcium, served to bring the colloidal clay medium to the same pH as the calcium. It is evident that the barium is not destructive to the bacteria if one judges by the number of legume bacteria that will grow in a clay saturated only with barium, as previously shown in Table 4.

To 25 ml. quantities of these media there were added 0.1 ml. of kraut juice and 0.25 grams of sucrose. They were sterilized, inoculated, aerated for three days; then loopful transfers were plated on calcium carbonate agar. The pH values of the media were determined before and after they were sterilized and after the cultures had grown. A series of three variant alfalfa cultures, V-2, V-10, V-11, and one normal alfalfa culture, N-5, were grown for four transfers in the no-calcium clay. The number of abnormal and normal colonies was counted and tabulated as percentages of the total; some abnormal colonies for the succeeding tests were picked from the no-calcium cultures to be held on agar slants. No attempt was made to save the colonies from the calcium clays. Transfers from the original stock served as inoculants in the succeeding trial. Cultures on the resulting slants as characteristic of the normal and variant forms were used to inoculate the next transfer to calcium and no-calcium clays. This process was repeated until the cultures had been grown in the clay four times.

The changes in the pH of the media and in the characteristics of the organism as the result of growth in the two media differing only in the presence or absence of calcium are recorded in Table 8 and Figure 9. It is apparent that even though the abnormal cultures were 100% abnormal at the outset, their growth in the barium clay resulted in a small part of the colonies appearing normal. This suggests that the barium clay suspension is more conducive to normal development than is the regular agar medium, where no such tendency to change toward normal was observed. In the absence of

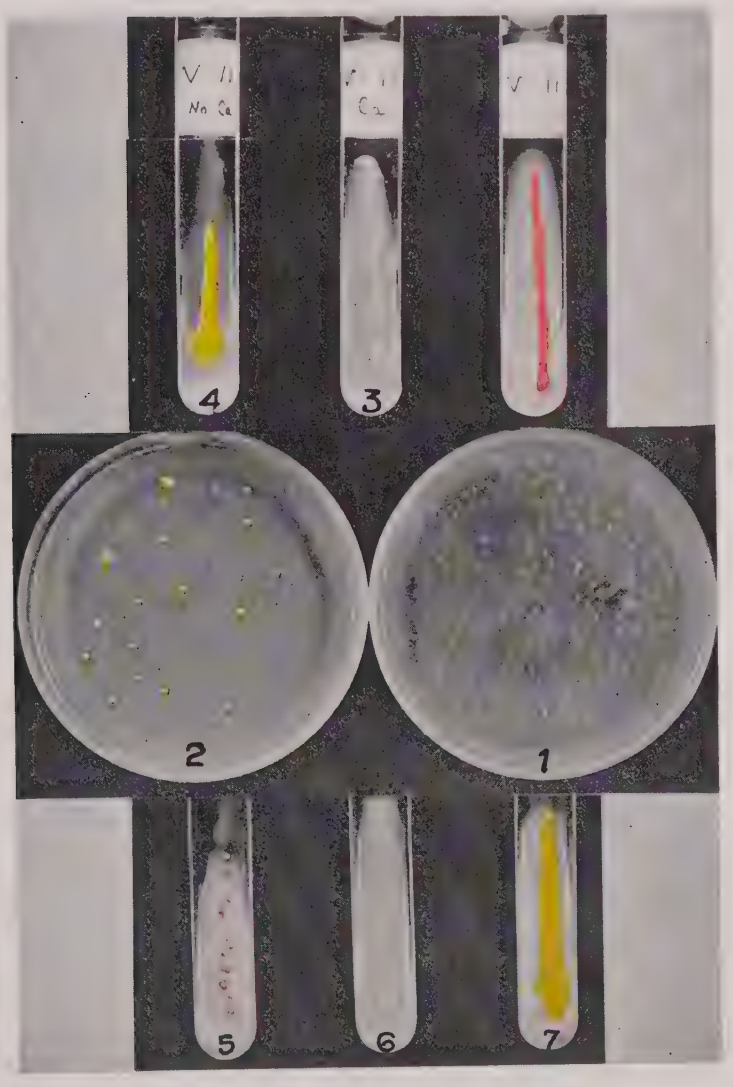


Plate A.—An abnormal (red) legume organism which was transformed to yellow on calcium deficient clay, and to white on calcium clay. Reversion from white to red was produced by calcium carbonate agar and restoration to white on calcium clay.

Plate B.—Abnormal organisms transformed to normal by calcium clay.

No. 1 Abnormal stock culture
No. 2 First transfer in no-Ca-medium
No. 3 First transfer in Ca-medium
No. 4 Fifth transfer in Ca-medium
No. 5 Plated on calcium carbonate agar from 2
No. 6 Plated on calcium carbonate agar from 4

PLATE C—Persistently abnormal (yellow) oil column remains (see with normal normality after adsorption on calcium carbonate clay

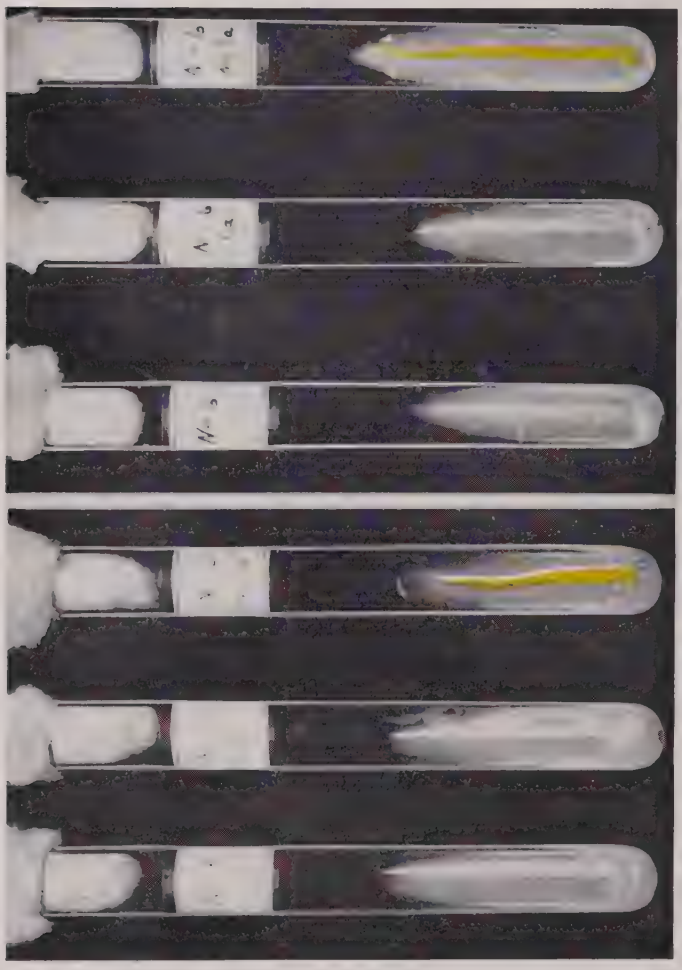


Plate D.—Normal legume organisms transformed to abnormal by calcium deficient clay. Left—alfalfa. Right—soybeans.

Plate E.—Nodulation differences due to calcium influence through the organisms and through the plant. Culture, N-5
Above—Plants inoculated with bacteria grown on Ca-clay
Below—Plants inoculated with bacteria grown on no-Ca-clay
Plants on left given calcium. Plants on right, no calcium

Plate F.—Nodulation differences due to plant calcium.
Above—Culture V-10. Below—Culture V-11
Left—Plants given calcium. Right—Plants, no calcium

TABLE 8.—THE BEHAVIOR OF LEGUME BACTERIA IN A NO-CALCIUM-BEARING MEDIUM—SET I.

Trial of Set	Culture No.	Inoculant Growth Condition	Growth Resulting % Normal		pH of Medium		
					Sterilization		After Colony Growth
			−Ca	+Ca	Before	After	
1	N-5	Normal	0	42	6.8 7.1	6.0 6.7	5.7 6.9
	V-10	Abnormal	0	20	6.8 7.1	6.7 6.7	6.9 6.7
	V-11	Abnormal	11	100	6.8 7.1	6.0 6.7	6.4 7.1
	V-2	Abnormal	32	40	6.8 7.1	6.0 6.7	6.3 6.9
2	N-5	Normal	41	100	7.9 7.4	7.5 7.1	6.8 6.4
	V-10	Abnormal	60	80	7.9 7.4	7.5 7.1	6.5 6.2
	V-11	Abnormal	66	98	7.9 7.4	7.5 7.1	6.2 6.2
	V-2	Abnormal	16	38	7.9 7.4	7.5 7.1	6.5 6.6
3	N-5	Abnormal	49	70	7.9 7.4	7.5 7.1	6.7 6.6
	V-10	Abnormal	0	6	7.9 7.4	7.5 7.1	6.5 6.7
	V-11	Abnormal	0	13	7.9 7.4	7.5 7.1	6.0 6.1
	V-2	Abnormal	0	17	7.9 7.4	7.5 7.1	6.4 6.3
4	N-5	Abnormal	0	6	7.9 7.4	7.5 7.1	6.3 6.4
	V-10	Abnormal	2	5	7.9 7.4	7.5 7.1	6.0 6.0
	V-11	Abnormal	0	0	7.9 7.4	7.5 7.1	6.1 6.1
	V-2	Abnormal	0	0	7.9 7.4	7.5 7.1	6.5 6.3

calcium, the normal organism gradually declined to abnormality so that at the conclusion of the four transfers no normal colonies appeared. The abnormal culture remained as such under the same conditions. It may be possible that the barium served to stimulate growth for a short time only. The pH values of the clay media were lowered after the variant and normal forms had grown in them. The cultural characters of those resulting from growth in the no-calcium media were as follows:

V-2 was a light yellow scant growth, very similar to the stock culture. The colonies were slightly raised and small.

V-10 was a deep yellow growth that accumulated at the bottom of the plant. Deep yellow raised colonies occurred on the plates.

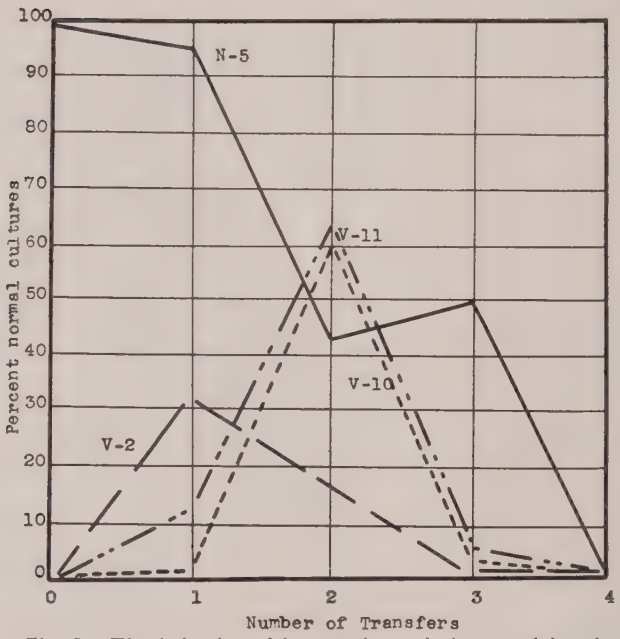


Fig. 9.—The behavior of legume bacteria in no-calcium-bearing medium.

V-11 also had a deep yellow growth. The colonies were raised and appeared of a jelly-like consistency.

N-5 was similar to the normal growth of the alfalfa culture, except that it had become yellow.

When the stock culture, V-11, a red variant alfalfa bacteria, was grown in the media bearing calcium and no calcium for three days, its cultural characters became somewhat modified. As a result of growth in the calcium-bearing medium all of the colonies appearing in plate (1) seemed to be normal and white, while a majority of those appearing in plate (2) from the growth in a no-calcium-bearing medium were yellow and abnormal. These differences are portrayed in Plate A (Page 21). Colonies picked from the plate containing the normal appearing culture and streaked on slants appeared white, as shown on slant (3). Colonies selected from plate (2) showing predominantly variants appeared yellow and abnormal, as represented by slant (4).

When some of the white colonies, however, were selected from plate (1) containing all normal appearing colonies, and put on calcium carbonate agar slants, they became red again, as shown on

slant (5). An additional passage of this culture through the calcium-bearing clay medium resulted in a normal white (6) growth with similarly normal cultural characters. Passage through the no-calcium-bearing medium gave yellow colonies as represented on slant (7).

The results indicate that pink colored colonies are a step nearer normal than are the yellow, since the culture became yellow when grown in the absence of calcium. Other data support the fact that yellow cultures sometimes give pink colored colonies in the presence of calcium.

Nutrients More Complete Including Calcium.—The *calcium*, potassium, magnesium and phosphate medium containing 0.25 grams of sucrose and 0.1 ml. of kraut juice per 25 ml. was autoclaved and aerated for three days after inoculation with the normal or abnormal cultures. Loopfuls were plated and the percentages of typical or normal and atypical or abnormal colonies were noted. Only the normal appearing organisms were selected from the agar plates made from those grown in the calcium clay. These normal organisms from this same source became abnormal in the no-calcium-bearing medium while they became more and more normal

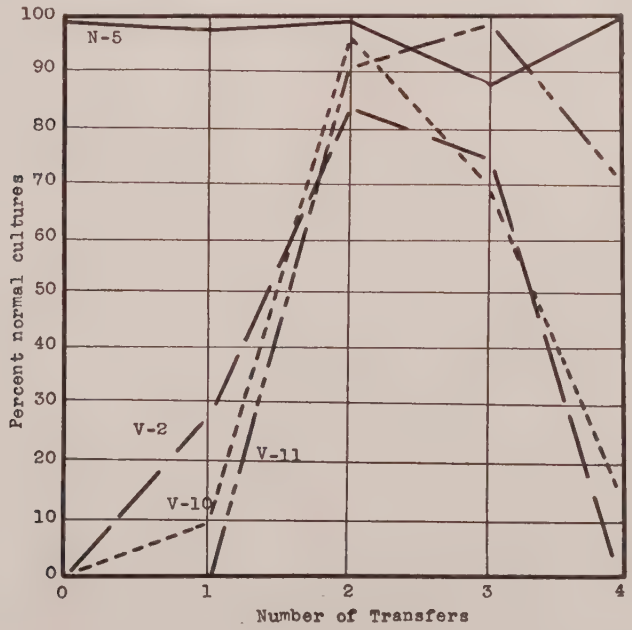


Fig. 10.—The transformation of abnormal legume organisms to normal on calcium clay.

TABLE 9.—GROWTH BEHAVIOR OF LEGUME BACTERIA IN A CALCIUM-BEARING MEDIUM—SET II.

Trial of Set	Culture No.	Inoculant Growth Condition	Growth Resulting % Normal		pH of Medium		
					Sterilization		After Colony Growth
			−Ca	+Ca	Before	After	
1	N-5	Normal	0	100	6.9 6.9	6.7 6.7	6.3 6.4
	V-10	Abnormal	0	10	6.9 6.9	6.7 6.7	6.0 6.1
	V-11	Abnormal	0	0	6.9 6.9	6.7 6.7	6.0 6.0
	V-2	Abnormal	3	38	6.9 6.9	6.7 6.7	6.0 6.0
2	N-5	Normal	62	100	7.0 6.7	7.4 7.0	6.8 6.6
	V-10	Normal	0	100	7.0 6.7	7.4 7.0	6.4 6.5
	V-11	Normal	0	100	7.0 6.7	7.4 7.0	6.3 6.4
	V-2	Normal	67	85	7.0 6.7	7.4 7.0	6.7 6.6
3	N-5	Normal	34	86	7.0 6.7	7.4 7.0	6.2 6.2
	V-10	Normal	60	68	7.0 6.7	7.4 7.0	6.3 6.1
	V-11	Normal	63	100	7.0 6.7	7.4 7.0	6.3 6.0
	V-2	Normal	77	77	7.0 6.7	7.4 7.0	6.3 5.9
4	N-5	Normal	53	100	7.1 7.5	7.1 7.4	6.8 6.9
	V-10	Abnormal	0	13	7.1 7.5	7.1 7.4	6.8 6.9
	V-11	Abnormal	0	68	7.1 7.5	7.1 7.4	6.8 7.3
	V-2	Abnormal	0	0	7.1 7.5	7.1 7.4	6.6 7.3

in the calcium-bearing medium. On this medium the normal alfalfa organism, N-5, remained normal both in color and cultural character. At the end of the second and third transfer in it the three variants appeared to be 100% normal. After the third transfer these cultures were held for two months on the calcium carbonate agar and two of the variants, V-2 and V-10, regained their yellow color. Another transfer caused them to lose some of their color and become more normal in appearance although they were not restored to normality. An additional transfer of V-10 in a calcium-bearing medium restored it to normality. Culture number V-2 never became normal. The trend of the percentages of normal and abnormal cultures resulting from growth in this medium is given in Table 9 and Figure 10.

The following are the characteristics of the cultures resulting from growth through four transfers or more in the calcium-bearing medium:

V-2 had a thin growth of light yellow color.

V-10 gave a white normal growth, but not as heavy as the normal alfalfa.

V-11 was a white, normal growth.

N-5 was a typically white, heavy growth characteristic of the alfalfa culture.

When the variant alfalfa stock culture, V-10, was grown in no-calcium medium it remained yellow, while growth for one transfer in the calcium-bearing medium resulted in an orange growth. The fifth transfer in the calcium-bearing medium resulted in white and normal colonies. The colonies of the culture from the no-calcium medium were yellow in the calcium carbonate medium; under similar conditions for the calcium medium the colonies were white and normal. The transformation of the organism is shown in Plate B (Page 22).

According to these results it seems that an orange colored culture is an intermediate variant between white and yellow. It also appears that yellow colored variants are about as highly abnormal as variants may become under the conditions imposed. It is also evident that the presence of calcium functioned in transforming the abnormal to normal cultures and that in the absence of calcium the variants remained abnormal.

One of the three variants of alfalfa, V-2, which was grown in the calcium-bearing medium for four transfers, did not become normal. Induced reversion was possible, but it appeared to be transitory in nature. When the stock culture was grown in the calcium-bearing medium for three days and then plated, approximately one-half of the colonies were yellow and abnormal, while the remaining colonies appeared normal. Selection of the white normal colonies, transplanting them to calcium carbonate agar slants, and allowing them to incubate for a few days induced the cultures to become yellow again. When the yellow cultures on the slants were again plated without growing in the calcium clay, all the colonies appeared to be abnormal and yellow. Results are shown in Plate C (Page 23).

The previous data have shown the restoration of the variants cultures to normal forms by supplying calcium liberally. In order to study the effect of the presence and absence of calcium on normal forms as an indication of the significance of calcium for legume organisms, the normal alfalfa culture, N-5, was carried through

the calcium and no-calcium medium. The stock culture of this was a heavy white grower and remained in this condition when grown in calcium clay medium. On the other hand, when calcium was absent from the clay medium, the organism became yellow and abnormal.

A normal soybean culture, N-6, portrayed the same changes. The stock culture, normal and white, remained normal after growth in a clay containing calcium, but growth in a clay medium in the absence of calcium resulted in the culture becoming abnormal. Observation of variants produced from the normal cultures showed that the characters were similar to those of the stock culture except for the yellow color. The stock, calcium and no-calcium cultures are shown in Plate D (Page 24).

These data indicate the significance of calcium. When it was absent, abnormal forms resulted. The colloidal clays seem especially superior to the regular calcium carbonate agar, not only in maintaining normal forms but in inducing the variants to become normal. In other words, it seems that the calcium clay complex delivers calcium in larger amounts and supplies it through some mechanism that is especially effective in the nutrition of the legume organism in making it grow more rapidly and develop into the white raised colony form which is considered as the normal growth.

Nodulation of the Host by Transformed Organisms.—The induced transformation of the variants to normal and vice versa, as judged by observable colony or laboratory characteristics, requires nodulation tests for confirmation. Since the legume bacteria were responsive to calcium treatments it seemed appropriate to observe the effect of calcium, supplied through the host, on nodulation by the bacteria from the previous transformations and treatments. As a check on the influence by calcium through the host, the test plants were grown with and without calcium. All cultures were tested in pots in triplicate with white sand as a medium. This had been leached previously with 1:1 HCl and washed free of chlorides with distilled water. Calcium was supplied in Crone's nutrient solution and also as calcium carbonate. Sodium was substituted for calcium in the nutrient solution containing no calcium. The seeds were sterilized in 1: 500 mercuric chloride.

Since each alfalfa culture was grown for four transfers in a medium containing calcium and no calcium, these bacteria were designated as the "calcium" and "no-calcium" bacteria, respectively. These cultures were then used to inoculate the host, with and without calcium. The averages of the nodule counts for triplicate pots of 25 plants per pot are listed in Table 10 and given graphically in Figure 11.

TABLE 10.—NODULATION OF ALFALFA AS PROVOKED BY CULTURES FROM CALCIUM AND NO-CALCIUM MEDIA.

		Ca Watered		No Ca Watered	
		Ca-Bact.	No-Ca-Bact.	Ca-Bact.	No-Ca-Bact.
N-5	Plants	42	47	52	54
	Nodules	116	112	97	26
	Average per plant	2.50	2.39	1.87	.48
V-10	Plants	58	60	53	61
	Nodules	180	52	42	23
	Average per plant	3.10	.87	.79	.21
V-11	Plants	83	76	66	79
	Nodules	275	127	2	28
	Average per plant	3.32	1.69	.03	.35
V-2	Plants	66	75	72	79
	Nodules	68	72	57	0
	Average per plant	1.03	.96	.79	0
Ck.	Plants	73	----	87	----
	Nodules	123	----	33	----
	Average per plant	1.68	----	.38	----

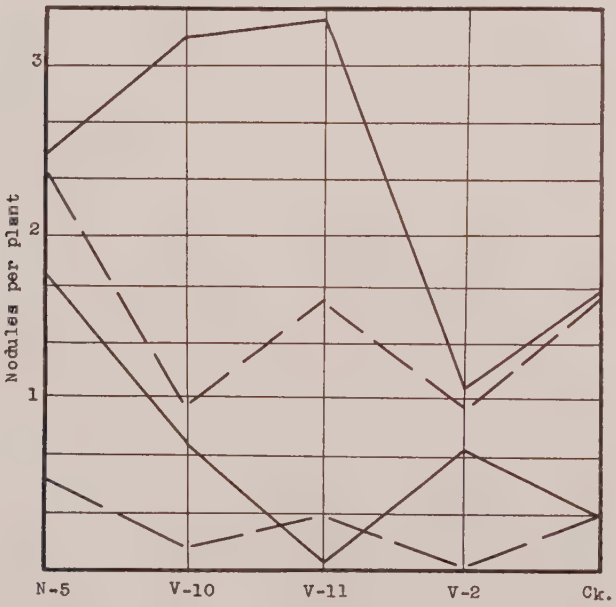


Fig. 11.—Nodulation of alfalfa by transformed legume bacteria.

Upper two curves show results from plants supplied with calcium; the lower two curves, plants without calcium.

———— "calcium" bacteria

— — — — — "no-calcium" bacteria

Some of the colonies, originally from the culture V-10, which had appeared as normal after growth in the calcium-bearing medium were used to inoculate the host. Some contamination occurred in the check pots. This was rather localized and its scattered nature indicated that it was contamination. The two variants, V-10 and V-11 which had been transformed to normality, gave even better nodulation of the host than the normal alfalfa culture. Also, the best nodulation resulted when calcium was supplied to both the bacteria before inoculation and to the host during growth.

In the second nodulation trial, at the end of the fourth transfer on the calcium clay which used the culture originally from V-10, the bacteria were abnormal and did not give nodulation. The presence of calcium in the host did not seem sufficient to cause it to nodulate. The culture V-11, which had become normal on growth in the calcium clay, gave good nodulation on the plants which received calcium. On the other plants there was no nodulation. The culture V-2 did not give nodulation under any circumstances. The culture N-5, whose organism remained normal on calcium clay and became abnormal on the no-calcium medium, gave good nodulation on plants supplied with calcium. The "calcium" and "no calcium" bacteria gave very poor nodulation on plants not supplied with calcium. Calcium seemed most effective as an influence on nodulation when supplied both through the plant and through the bacteria. The results of the second trial are given in Table 11 and the nodulation behavior of N-5 is illustrated in Plate E (Page 25).

The third nodulation trial included an abnormal alfalfa culture which had been transferred from the normal, N-1, to an ab-

TABLE 11.—NODULATION OF ALFALFA WITH CULTURES GROWN IN CALCIUM AND NO-CALCIUM BEARING MEDIA.

		Plants Supplied with Ca		Plants not Supplied with Ca	
		Ca-Bact.	No-Ca-Bact.	Ca-Bact.	No-Ca-Bact.
N-5	Plants	69	75	90	51
	Nodules	277	262	50	6
	Average per plant	4.01	3.47	.55	.11
V-10	Plants	64	75	75	42
	Nodules	0	69	11	0
	Average per plant	0	.92	.14	0
V-11	Plants	81	85	57	73
	Nodules	265	.03	0	0
	Average per plant	3.26	.03	0	0
V-2	Plants	84	57	86	77
	Nodules	4	2	0	0
	Average per plant	.04	.04	0	0
Ck.	Plants	159	----	114	----
	Nodules	37	----	0	----
	Average per plant	.20	----	0	----

normal by growth in a clay medium bearing potassium. The organism remained normal in the calcium medium. The bacteria grown in the calcium clay gave good nodulation while the "no calcium" bacteria did not give nodulation. The normal culture which had been completely transformed to normal from the abnormal V-10 by a fifth transfer in calcium medium gave good nodulation in the calcium-bearing plant. The normal culture, N-5, gave good nodulation in the calcium-bearing plants, as shown in Table 12 and illustrated in Plate F. (Page 26).

TABLE 12.—NODULATION OF ALFALFA PLANT GROWN WITH AND WITHOUT CALCIUM BY CALCIUM AND NO-CALCIUM BACTERIA.

Culture No.		Plants Supplied with Ca		Plants not Supplied with Ca	
		Ca-Bact.	No-Ca-Bact.	Ca-Bact.	No-Ca-Bact.
N-1	Plants	56	70	80	57
	Nodules	129	0	70	0
	Average per plant	2.30	0	.87	0
N-5	Plants	44	63	45	70
	Nodules	128	132	47	18
	Average per plant	2.93	2.10	1.04	.25
V-10	Plants	59	73	70	63
	Nodules	137	0	0	0
	Average per plant	2.32	0	0	0
Ck.	Plants	101	----	90	----
	Nodules	0	----	0	----
	Average per plant	0	----	0	----

As a test of the influence of calcium in the medium on the inoculating capacity, a common source of a normal organism, N-6, was used and maintained normal by four transfers through the "calcium" medium and transformed to abnormal by similar culture through a "no-calcium" medium, as is shown in Plate D (Page 24). The "calcium" and "no-calcium" bacteria so produced were used to inoculate pans of the host plant, each pan containing ten plants. The height, nodules and weight of these soybeans are given in Table 13 and represented graphically in Figure 12. The "calcium' bacteria did not bring about nodulation in the absence of a calcium supply to the host but there was nodulation when calcium was supplied to the plant. The "no-calcium" bacteria failed to nodulate the plants whether or not they had been grown with calcium.

The height, weight, and number of nodules of the plants grown without calcium were similar in that there were no nodules produced, irrespective of whether they were inoculated with "calcium" or "no-calcium" bacteria. Both were greater than the check without bacteria. Although there were no visible nodules on the plants inoculated with the "no-calcium" bacteria, the weights of the plants

TABLE 13.—THE NODULATION, GROWTH AND WEIGHT OF SOYBEANS INOCULATED WITH "CALCIUM" AND "NO-CALCIUM" BACTERIA

		N-6 "Calcium" Bacteria	N-6 "No-calcium" Bacteria	Check
Plants supplied with Calcium	Height (cms.)	45	37.0	40.0
	Nodules	66.0	0.0	0.0
	Weight per 20 plants (grams)	15.75	15.71	11.20
Plants not supplied with Calcium	Height (cms.)	10.0	8.0	6.5
	Nodules per 20 plants	0.0	0.0	0.0
	Weight per 20 plants (grams)	7.32	7.10	7.00

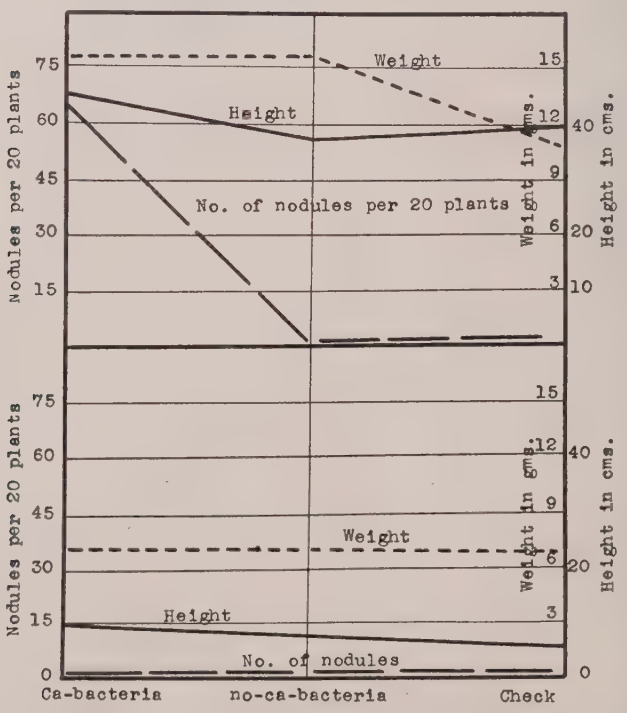


Fig. 12.—Plant performance in the presence or absence of calcium, as related to inoculation by "calcium" and "no-calcium" bacteria.

Upper graph—Plants given calcium
Lower graph—Plants not given calcium

were equal to those of the nodulated plants. This would suggest that the presence of legume bacteria at the roots of the hosts has benefitted them and increased their growth, a suggestion in agree-

ment with past observations[31], in which it was concluded that the legume bacteria may benefit the host without nodulation.

When the same soybean culture, N-6, was grown in a potassium-bearing medium in the absence of calcium, it became yellow and abnormal. The use of this culture to inoculate in the presence and absence of calcium, failed to produce nodules. However, the use

TABLE 14.—THE NODULATION, GROWTH AND WEIGHT OF SOYBEANS INOCULATED WITH "CALCIUM" AND "NO-CALCIUM" BACTERIA.

		"Calcium" Bacteria	"No-calcium" Bacteria	Check
Plants supplied with Calcium	Height (cms.)	37.0	35.5	34.0
	Nodules	197.0	0.0	0.0
	Dry Weight (grams)	3.578	3.720	2.962
Plants not supplied with Calcium	Height (cms.)	18	22.5	18.5
	Nodules	9.0	4.0	0.0
	Dry Weight (grams)	2.342	2.720	2.856

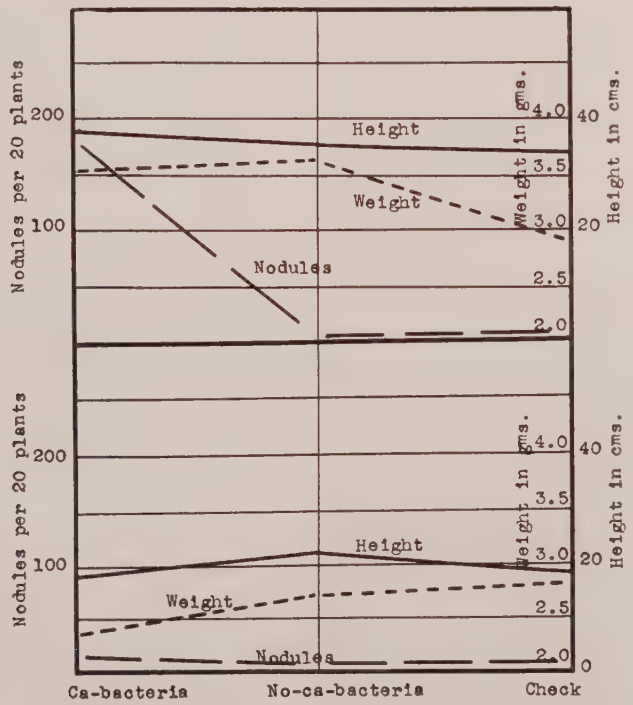


Fig. 13.—Plant performance in the presence or absence of calcium as related to inoculation by "calcium" and "no-calcium" bacteria.

Upper graph—Plants given calcium

Lower graph—Plants not given calcium

of this same organism, grown in a clay medium bearing calcium, where it remained normal, produced a large number of healthy nodules clustered about the base of the tap root. The results which are given in Table 14 and represented graphically in Figure 13, show that potassium does not substitute for calcium in the clay medium.

In agreement with preceding results, it was observed that the weight of the plants receiving calcium and inoculated with "calcium" and "no-calcium" bacteria corresponded closely, while the check receiving no bacteria was considerably less. This again emphasizes the fact that the presence of the bacteria on the roots of the host may have benefitted it even though nodules were not produced.

DISCUSSION AND SUMMARY

It appears that the absence of calcium is responsible for the appearance of abnormal bacteria from normal forms. It would seem from the nature of legumes, which require a large amount of available calcium for their growth, that during the process of evolution Nature would likewise fit or necessitate a similar condition for the legume bacteria. The results of this study confirmed the contention that the customary medium employed for culturing legume bacteria has been deficient in an adequate supply of an element necessary for the most proficient growth of the organism.

Non-Leguminous Plants as Unsuited Media for Legume Bacteria

There has been no conclusive evidence that legume bacteria will infect plants other than the legumes. In the light of the results of this study, the nutritional fitness of the legume plants as a host or as a medium for bacteria is suggested, as based on the fact that legumes are usually higher in calcium than are non-legumes. This difference between the two might seem a possible reason for the difference as to infection by nitrogen-fixing bacteria and would seem to indicate that the non-legumes could not be infected by the legume bacteria because their calcium supply is too low. Even if all other physiological conditions were favorable for the nodulation the low calcium content might prohibit infection. The calcium content of non-legumes is approximately one-third to one-fifth of that of the legumes, as given by Fred, Baldwin and McCoy[23], (other papers are cited by them).

The Mechanism of Ion Exchange in the Assimilation of Mineral Nutrients by Legume Bacteria

According to the results of this study, the pH of the medium dropped considerably after bacterial growth; the decrease in pH figures varied with a range from 0 to 1.2 pH units. This change means that the bases were removed from the clay particle and hydrogen substituted for them.

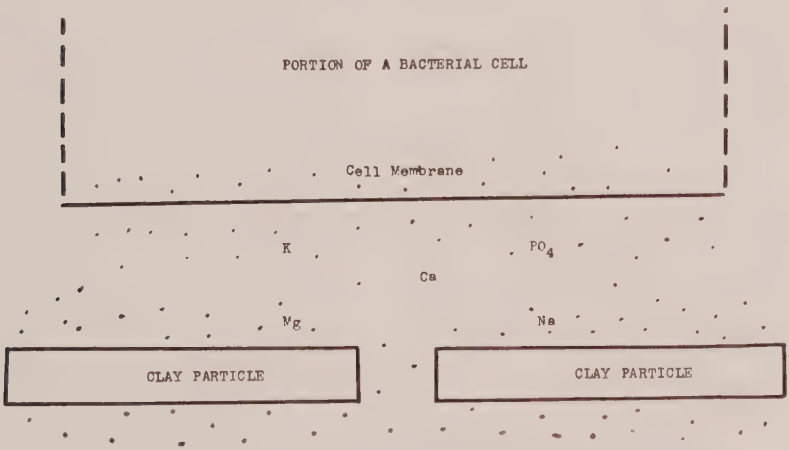


Fig. 14.—Mechanism of ion exchange in assimilation of mineral nutrients.

If we assume that the legume bacteria are five microns long and the clay particles 10 millimicrons in length, then the bacteria have a length 500 times that of the clay particles. If, as is generally assumed, the clay particles are plates, and the soil system consists of particles with 10 millimicrons length, it would require 500 such plates to equal the length of the bacterium. The adsorbed ions on the surface of the clay particles would come in contact with the bacterial cell in a manner somewhat as represented in Figure 14.

Since the cell absorbs sucrose and oxides it with the release of water and carbon dioxide, the carbon dioxide reacts with water to form carbonic acid which ionizes to form bi-carbonate and hydrogen ions. The bacteria which have a negatively charged, semi-permeable membrane will adsorb the hydrogen ions. When the bacterial cell comes in contact with the clay particle having potassium, calcium, and other cations adsorbed and mobile, the adsorbed and mobile hydrogen ion of the bacterium may inter-change with the potassium of the clay particle. There is then an exchange and the hydrogen becomes absorbed on the clay particle while the

potassium becomes adsorbed on the bacterial cell. During rapid oscillations, the ion passes into the cell and becomes fixed as a part of the living matter constituting the cell.

Mechanism of Phosphate Fixation by Colloidal Clay

It was observed that the growth of the soybean organism, quite contrary to the effects by the alfalfa organism, caused the medium to become more alkaline. This would suggest that while the alfalfa bacteria take up the cations in larger quantities than the anion, these soybean bacteria act in the reverse order and take up the anion in larger amounts than the cations, including the calcium. It seems evident that if the calcium is connected between the clay and phosphate anion that when the anion is taken in larger amounts than the calcium cation, this will leave excess calcium which will form calcium hydroxide and give a more alkaline reaction. It was observed that the medium became more acid by the growth of the soybean culture in a clay medium containing only calcium. This indicated that the organism used calcium and substituted hydrogen which, with the phosphate ion, gave a more acid reaction.

In order to test the validity of this hypothesis, a culture of alfalfa, N-5, of soybean, N-6, and a check without bacteria were carried through in the customary way. At the end of ten days a portion of each of the three was filtered through an ultrafilter and another portion was washed with N/1 potassium chloride three times, while the clay was removed by centrifuging. The amounts of calcium and phosphorus were determined in the clear solutions obtained in these two methods of treatment. On the original clay as prepared and previously outlined in Figure 3, there were adsorbed 0.6 M.E. of calcium and 0.2 M.E. of phosphorus. The following data in Table 15 show the different amounts of calcium and phosphorus removed by the treatments after the growth of the alfalfa and soybean organisms for this time period.

TABLE 15.—THE REMOVAL OF CATIONS AND ANIONS BY WASHING AND EXCHANGE AFTER BACTERIAL GROWTH.

	pH of Medium			Treatment	M.E. of Calcium	M.E. of Phosphorus
	Sterilization		After Colony Growth			
	Before	After				
Check	6.9	6.95	6.9	KCl	.495	None
				Filtered	.088	.00092
N-5 Alfalfa	6.9	6.95	6.7	KCl	.517	.00338
				Filtered	.144	.000367
N-6 Soybean	6.9	6.95	7.0	KCl	.510	.00438
	---	----	---	Filtered	.449	None

The large amount of free or ionized calcium in the filtrate of the soybean culture, N-6, and the absence of ionized, or free, phosphorus is an interesting observation; likewise the low amount of ionized, or free, calcium in the filtrate of the alfalfa culture, when in all three tests the calcium exchanged by potassium chloride was an almost constant amount. One might believe that by the former, N-6, or soybean bacteria, more of the anion, phosphate, was taken than of the cation, calcium, while in the latter, N-5, the alfalfa bacteria consumed more of the cation than of the corresponding anion phosphate. This would seem possible if these two, calcium and phosphorus, were adsorbed on the clay as a calcium phosphate compound, or with the phosphorus linked through the calcium. It was observed also that the potassium chloride exchanged only about five-sixths of the added calcium in the clay and suggests that some of the calcium was held very firmly in the clay. If the reaction changes in the media were due to the differences in utilization of the calcium ion or of the phosphate ion by the alfalfa and soybean organisms, it suggests that the phosphorus may be held to the clay through the calcium linkage. Only carefully controlled physicochemical studies can fully explain the chemical conditions suggested by these biological phenomena.

Colloidal Clay as a Medium

The colloidal clay particles represented as plates have the various mineral nutrients adsorbed on the surface where concentration may permit use by the bacteria. Thus the chances for a bacterium to get its mineral needs are greatly enhanced by the concentration at the surface. The enormous surface of the colloidal particle may also speed bacterial metabolism by greatly increasing the intake of mineral and organic needs and by helping the cell to eliminate the waste products.

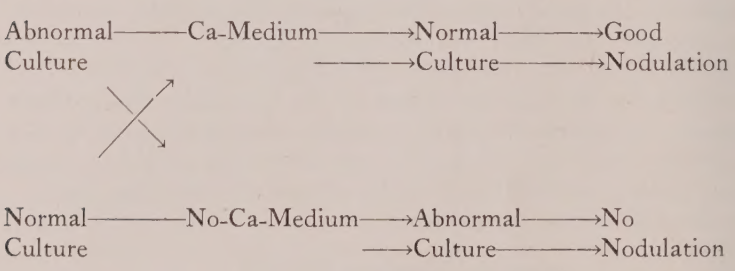
Bacteria are colloidal bodies with enormous surfaces and consequently require a great deal more energy than do living bodies with less outer surface.[29] It is possible that the colloidal clay particles with the nutrients adsorbed on the surface materially decrease the energy required to grow the organism in the customary nutrient solution.

The results confirm the theory that calcium is responsible for controlling the behavior of legume bacteria. The abnormal forms sometimes occuring in calcium carbonate media may be caused by a deficiency of available calcium. When calcium is made available in larger amounts through the colloidal clay complex these abnormal forms become normal. Other cations serve as nutrients but

must not be present in such quantities as calcium. The order of suitability as a nutrient appears to be Ca-Mg-Ba-K-H in producing numbers and in controlling the degree of normal bacteria in a culture. The results substantiate the fact that hydrogen is not injurious because the legume bacteria may make excellent growth at a low pH if the proper cations, especially calcium, are present.

Calcium probably functions as a nutrient and as an agent responsible for a physiological balance of other nutrients.

The results show that calcium may change the abnormal forms of legume bacteria to the normal forms. Furthermore, in the absence of calcium the abnormal forms will remain abnormal and the normal will become abnormal. This may be illustrated by the following diagram.



CONCLUSIONS

1. A bacteriological medium was prepared by use of electrodialyzed colloidal clay which simulated soil conditions that were chemically and bacteriologically controllable. The experimental conditions imposed were thus very similar to soil conditions.

2. In the presence of an ample supply of calcium, normal legume bacteria remained normal and abnormal or so-called variant forms became normal. All of these, after growth with ample calcium, gave good nodulation on plants supplied with calcium.

3. In the absence of calcium, the normal legume bacteria became abnormal and the abnormal forms remained abnormal; both failed to nodulate the host plant.

4. The best nodulation was secured when both the bacteria and the plants were given calcium.

5. No nodulation occurred when neither the bacteria nor the plants received calcium.

6. The order of importance as nutrients for legume bacteria was suggested as follows: Ca-Mg-Ba-K-H.

7. Growth of the bacteria was related to the amount of calcium presented in the media.

8. For the best growth of the legume bacteria it was necessary to have the clay at least 50% saturated with calcium.

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